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Dynamics of Bacterial and Fungal Communities on Decaying Salt Marsh Grass†

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Both bacteria and fungi play critical roles in decomposition processes in many natural environments, yet only rarely have they been studied as an integrated microbial community. Here we describe the bacterial and fungal assemblages associated with two decomposition stages of *Spartina alterniflora* detritus in a productive southeastern U.S. salt marsh. 16S rRNA genes and 18S-to-28S internal transcribed spacer (ITS) regions were used to target the bacterial and ascomycete fungal communities, respectively, based on DNA sequence analysis of isolates and environmental clones and by using community fingerprinting based on terminal restriction fragment length polymorphism (T-RFLP) analysis. Seven major bacterial taxa (six affiliated with the α-*Proteobacteria* and one with the *Cytophagales*) and four major fungal taxa were identified over five sample dates spanning 13 months. Fungal terminal restriction fragments (T-RFs) were informative at the species level; however, bacterial T-RFs frequently comprised a number of related genera. Amplicon abundances indicated that the salt marsh saprophyte communities have little-to-moderate variability spatially or with decomposition stage, but considerable variability temporally. However, the temporal variability could not be readily explained by either successional shifts or simple relationships with environmental factors. Significant correlations in abundance (both positive and negative) were found among dominant fungal and bacterial taxa that possibly indicate ecological interactions between decomposer organisms. Most associations involved one of four microbial taxa: two groups of bacteria affiliated with the α-*Proteobacteria* and two ascomycete fungi (*Phaeosphaeria spartinicola* and environmental isolate "4clt").

Southeastern U.S. coastal salt marshes are among the most productive ecosystems known (24, 31) and provide a model environment for investigating detritus-based ecosystems and their decomposer communities. In these systems, both fungi and bacteria are recognized as key components of the decomposer community (1, 26), providing primary links in the remineralization and transformation of decaying vascular plant material.

In the few studies that have considered the activity of salt marsh bacterial and fungal saprophytes simultaneously, interactions between fungi and bacteria have been hypothesized to be based on temporal resource partitioning (29). According to this view, fungal colonization of senescing salt marsh cord grass (*Spartina alterniflora*) mediates the initial transformation of organic matter through extracellular enzyme activity and physical disruption (24, 26). In the fungus-dominated stage, *Spartina* undergoes loss of up to 60% of the original organic mass (26). As decomposition proceeds, the blades gradually collapse onto the marsh sediment and are reduced to smaller pieces with larger surface areas. Bacterial standing crop gradually increases, and bacteria assume a more prominent position in the latter stages of the decomposition process (1, 29).

This view of temporally segregated fungal and bacterial decomposition in salt marshes may be overly simplistic, however. Metabolically active bacteria and fungi have been shown to co-occur on *Spartina* detritus for much of the decomposition process (27). Furthermore, there has been no satisfactory explanation of the mechanisms by which microbial communities are replaced during temporal resource partitioning. A broader view that recognizes the potential for physiological and ecological interactions between co-occurring bacterial and fungal groups may be a more valuable perspective for addressing the fate of vascular plant-derived organic matter in coastal ecosystems (21).

Two prerequisite steps for investigating the roles of bacterial and fungal communities on vascular plant detritus are knowledge of the taxonomic composition of each community and an understanding of the patterns of occurrence of individual taxa. Previous studies of the fungal community of southeastern U.S. salt marshes have identified several species of ascomycetous fungi as major decomposers of *S. alterniflora* blades, based on both traditional culture- and microscopy-based methods (24–26) as well as molecular approaches (4). The two most prevalent and virtually omnipresent species are *Phaeosphaeria spartinicola* and *Mycosphaerella* sp. strain 2 (17), both of which are involved in lysis of lignocellulosic components of the blades (2, 26, 28). Additional species that are typical but less prevalent members of the community (occurring in <40% of blades examined) include *Phaeosphaeria halima*, environmental isolate "4clt" (an ascomycetous species that does not yet have a formal taxonomic description; see reference 4), and *Buergenerula spartinae* (4, 25). Species of mitosporic fungi (i.e., species that are probably asexual forms of ascomycetes) have also been detected in decaying blades (17).

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In contrast, relatively little is presently known of the composition of the bacterial community associated with the *S. alterniflora* decay system. Culturing approaches are generally not successful for identifying ecologically dominant bacterial species in marine environments (8, 13), and molecular methods for assessing community composition have seldom been brought to bear on the bacterial members of the salt marsh decay system. However, genes encoding aromatic ring-cleaving dioxygenases are common among the bacteria colonizing decaying *S. alterniflora*, and these genes appear to come primarily from α-*Proteobacteria* (5).

In this study, we use molecular, culture, and microcopy-based techniques to simultaneously examine the fungal and bacterial communities associated with *S. alterniflora* blades, describing the composition and dynamics in three replicate plots for two stages of decay at five time points over a 13-month period.

MATERIALS AND METHODS

Site description and sample collection. Decaying blades of tall-form *S. alterniflora* were collected from Dean Creek Marsh, Sapelo Island, Ga., in July 2000 (Jul-00), October 2000 (Oct-00), January 2001 (Jan-01), April 2001 (Apr-01), and July 2001 (Jul-01). Dean Creek Marsh is a Georgia Coastal Ecosystems Long Term Ecological Research sampling area and a Sapelo Island Microbial Observatory sampling area and is typical of southeastern U.S. salt marshes (6) (maps available at http://gce-lter.marsci.uga.edu/lter/asp/studysites.htm). Senescent blades that represented two distinct temporal stages of decomposition were collected: "early-decay" blades were yellow or brown in color, remained attached to the stem, and were not yet collapsed onto the sediment; "late-decay" blades were brown to black in color and also remained attached to the stem, but were collapsed onto the sediment surface.

Three 5-m-diameter replicate plots separated by 15 m (designated plots 1 to 3) were established in Dean Creek Marsh, and 32 blades from each category were collected from each plot (6 total samples). For each sample: (i) 10 6-cm blade samples were cut in half, with one portion used for fungal isolation, biomass measurements, and microscopy and the other used for DNA extraction; (ii) 12 4-cm pieces were used to measure rates of microbial respiration (plot 1 only); and (iii) 10 3- to 6-cm blades were assayed for carbon, hydrogen, and nitrogen content by using a Perkin-Elmer 2400 CHN analyzer (Wellesley, Mass.) at the University of Georgia's Chemical Analysis Laboratory.

Fungal observations and isolations. Occurrence of ascomycetes in decaying leaf blades was recorded by observation of spore-capture coverslips and direct microscopy of leaf surfaces (25). Briefly, the 3-cm lengths of blades were rinsed in tap water, with gentle rubbing to remove clay films, and then soaked for 10 min in tap water. Each wet blade piece was positioned within a 60-by-15-mm glass dish, 7 mm above a clean coverslip (6.25 cm^2), with the abaxial surface facing the coverslip. The dish contained deionized water in the bottom to maintain 100% relative humidity, and all dishes were enclosed within a 4-liter sealed plastic bag, along with an open dish of deionized water to prevent any drying of incubation dishes. Dishes were incubated at 20°C, under 30 $\mu E\ m^{-2}\ s^{-1}$ photosynthetically available radiation (12 h on/12 h off) for 72 h. Three 3-cm pieces were incubated for each of the blade types (early and late decay) from each of the three marsh plots at the Dean Creek site.

Each coverslip was subsequently examined under the dissecting microscope (Wild M8) at ×100 along its entire width below where the center of the blade piece had been positioned (25). In the first two samplings (Jul-00 and Oct-00), we visually estimated relative order of abundance of each spore type, from most frequent to least frequent. However, in the remaining samples (Jan-01, Apr-01, and Jul-01) specific ejection rates for each blade type were recorded. Species of ascospores were identified according to Kohlmeyer and Kohlmeyer (17), Kohlmeyer and Volkmann-Kohlmeyer (16), and Leuchtmann and Newell (19), using a ×400 Zeiss Standard 16 Research microscope with interference contrast. The abaxial surface of each blade piece was also examined under the dissecting microscope to check for the presence of ascomata of species that were not recorded as having expelled ascospores.

Fungal biomass measurements. Living fungal mass was measured as ergosterol content (22, 23) for all samples except Jul-00. Six 1.5-cm pieces of each blade type were pooled in a 20-ml screw-cap vial, 5-ml reagent ethanol was added, and the vial was stored at 4°C in the dark. Samples were subsequently reflux extracted in methanol, partitioned into pentane, and taken through high-performance liquid chromatography (HPLC) along with procedural standards of pure ergosterol as described by Newell (22, 23). A conversion factor of 200 U of fungal organic mass per U of ergosterol was used to calculate living fungal mass (23).

Microbial respiration rates. Microbial respiration rates were measured for both blade types of plot 1 samples by placing a 2-cm piece of blade into a 60-ml ashed biological oxygen demand (BOD) bottle and filling the bottle with filter-sterilized (0.2-μm pore size) Dean Creek water. Bottles were incubated underwater in the dark at 22°C in a water bath. Initial dissolved oxygen concentrations were determined by fixing three replicate bottles of each blade type with Winkler chemicals. At various time intervals over the following 24 h, three replicate bottles of each blade type were fixed. All fixed bottles were then titrated for determination of dissolved oxygen concentrations by using the precision Winkler method with automatic titration (32). Respiration rates (micromolar O_2 per square centimeter per hour) were calculated as the slope of the linear regression of oxygen consumed versus incubation time.

Bacterial cultures. Additional decaying blades (early and late stage) were collected from the Dean Creek site in May 2001 for bacterial isolations. These isolates were obtained by grinding blades in a sterile blender with filter-sterilized seawater and either spreading the liquid directly onto low-nutrient seawater plates (solid plates) or first mixing an aliquot with low-nutrient seawater medium containing 0.5% agar (semisolid plates) and pouring on top of solid medium (1.5% agar). Low-nutrient seawater medium contains (per liter) 10 mg of proteose peptone and 5 mg of yeast extract in filter-sterilized diluted Sargasso Seawater that has been aged for more than 1 year in the dark (final salinity, 24) (14). Representative isolates obtained by these approaches are designated first by the prefix "S" (standing blades in early decay) or "L" (lying blades in late decay) and either "H" (solid plates) or "S" (semisolid plates) followed by a numerical character.

Bacterial isolates were also obtained from ascomata found on late-decay blades collected during the Oct-00 sampling. Aged empty ascomata from the fungi *Phaeosphaeria spartinicola* and *Buergenerula spartinae* were picked from the decaying blades and dragged across a dilute V8 agar plate (DV8; 2 ml of V8 [Campbell Soup, Inc.], 20 g of agar in 1 liter of half-strength seawater [15 g of sea salts $liter^{-1}$]). Bacterial isolates obtained in this manner were designated by the prefix "Pspc" or "Bs" to indicate they were cultivated from a *P. spartinicola* or *B. spartinae* ascomata, respectively.

DNA extractions and PCR amplifications for clone libraries. DNA was obtained from bacterial and fungal isolates by using either cultures scraped from plates (bacteria and yeasts) or mycelia (fungi) using soil DNA extraction kits (MoBio, Solana, Calif.). DNA was obtained from 10 3-cm decaying *S. alterniflora* blades using Mega Size soil DNA kits (MoBio). Bacterial 16S rRNA genes were amplified with general bacterial primers 8F (5′-AGAGTTTGATCMTGGCTCAG-3′, where M is A or C) and 1522R (5′-AAGGAGGTGATCCANCCRCA-3′, where N is A, T, C, or G and R is A or G) (12). Fungal internally transcribed spacer (ITS) regions (3, 11) were amplified with the ascomycete-specific primers ITS1F (5′ CTTGGTCATTTAGAGGAAGTAA 3′) and ITS4A (5′ CGCCGTTACTGGGGCAATCCCTG 3′) (18). These primers amplify a product of ~600 bp, including the ITS1, 5.8S, and ITS2 regions of the rRNA operon. A previous study found no evidence for intraorganismal variation in ITS sequences for the common salt marsh fungi (4). All PCRs were carried out with Ready-To-Go PCR beads (Amersham Pharmacia, Piscataway, N.J.) with 0.2 μM each primer and 50 ng of DNA. Thermal cycling reactions for 16S rRNA gene amplification began with an initial 3 min at 95°C, followed by 25 cycles of 1 min at 95°C, 1 min at 60°C, and 1.5 min at 72°C. Conditions for ITS amplifications consisted of an initial 3 min at 95°C, followed by 35 cycles of 1 min at 95°C, 30 s at 52°C, and 1 min at 72°C. For both cycling reactions, a final step of 10 min at 72°C was included to complete any partial polymerizations. Products of the appropriate size were recovered from the gel with a QiaSpin gel extraction kit (Qiagen, Valencia, Calif.), and the PCR products were cloned by using a TA cloning kit (Invitrogen Corp., Carlsbad, Calif.).

Sequencing and phylogenetic analysis. Approximately 400 bp of sequence information was obtained for the 16S rRNA genes or ITS region sequences of bacterial and fungal isolates and environmental clones by either directly sequencing the PCR product following purification with an Ultra Clean PCR Clean-Up kit (MoBio) (isolates) or sequencing purified plasmid DNA (clones) using either the 8F (bacterial) or ITS1F (fungal) primer on an ABI PRISM 310 (Applied Biosystems). Sequences were analyzed by using the Wisconsin Package 10.0 (Accelrys, Burlington, Mass.). Phylogenetic trees were constructed with the PHYLIP package by using evolutionary distances (Jukes-Cantor) and the neighbor-joining method (10).

TABLE 1. Biological and chemical characteristics of early- and late-decay blades, and environmental characteristics of the Sapelo Island salt marsh ecosystem from July 2000 through July 2001[a]

Sample date (mo-yr) and decay stage	Organic density (mg of OM cm^{-2})	% Ash	C/N ratio	Respiration ($\mu M\ cm^{-2}\ h^{-1}$)	Ergosterol ($\mu g\ g\ of\ OM^{-1}$)	Ascospore expulsion (spores $cm^{-2}\ h^{-1}$)	Average monthly max temp (°C)	30-yr average monthly max temp (°C)	Cumulative monthly rainfall (cm mo^{-1})	30-yr cumulative monthly rainfall (cm mo^{-1})
Jul-00										
Early	12.3 (0.7)	20.2 (2.8)	60.7 (25.8)	0.87	ND	ND				
Late	9.7 (1.5)	40 (2.4)	51.1 (8.6)	0.49	ND	ND	33.2	32.2	6.4	16.5
Oct-00										
Early	13.7 (0.6)	13.6 (1.3)	69.4 (20.2)	0.68	288 (12)	ND				
Late	10.4 (0.9)	34.6 (1.9)	37.8 (5.2)	1.64	292 (54)	ND	25.2	25.5	0.3	8.9
Jan-01										
Early	13.5 (2.2)	13.1 (1.9)	80.7 (17.9)	0.68	463 (137)	237 (70)				
Late	11.2 (1.3)	40 (3.8)	64 (17)	1.4	265 (47)	103 (89)	14.3	16.1	3.5	10.0
Apr-01										
Early	14.3 (1.9)	10.1 (2.6)	53.9 (10.5)	3.15	685 (197)	1,394 (663)				
Late	9.9 (0.4)	23 (2.7)	51.7 (4.2)	1.31	577 (23)	163 (52)	24.7	24.4	1.7	6.2
Jul-01										
Early	11.7 (0.8)	14.3 (1.8)	56.5 (1.5)	1.67	424 (90)	481 (362)				
Late	9.6 (0.9)	26.6 (0.9)	49.9 (1.5)	1.68	289 (67)	122 (107)	32.1	32.2	15.3	16.5

[a] Thirty-year average temperature and precipitation data are from Chalmers (6) ($n = 3$). Values in parentheses represent ±1 standard deviation. ND, not determined; OM, organic mass.

Microbial community and isolate characterization using T-RFLP analysis. Terminal restriction fragment length polymorphism (T-RFLP) analysis (20) was carried out as follows. PCR amplification was carried out as described above with the exception that the ITS1F or 8F primers were fluorescently labeled on the 5′ end with FAM (carboxyfluorescein). Products were recovered from a 1.0% agarose gel with the QiaSpin gel extraction kit (Qiagen). Restriction enzyme digestion of the PCR product was carried out in a 10-μl total volume containing either 100 ng (community) or 10 ng (isolate/clone) of purified PCR product and either 10 U of *Cfo*I (16S ribosomal DNA [rDNA] sequences) or 10 U of *Hae*III (ITS sequences) at 37°C for 3 h. Digested DNA was precipitated in ethanol and suspended in 12 μl of deionized formamide with 1 μl of DNA fragment length standard Gene-Scan-2500 TAMRA (tetramethylrhodamine; Applied Biosystems). The terminal restriction fragment (T-RF) lengths were determined on an ABI PRISM 310 in GeneScan mode.

T-RFLP output data were analyzed with a Visual Basic program that reconciles minor shifts in fragment sizes between successive chromatograms (33). Peaks comprising <1% of total chromatogram area were excluded from the analysis. Principal component analysis (PCA) was performed with The Unscrambler 6.11 software (Camo, Corvallis, Oregon).

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in GenBank under accession no. AF460868 to AF460962, AY149733 to AY149815, AY149621 to AY149628, AY095104, and AY363101.

RESULTS

Environmental setting. Environmental variables and microbial activity levels were monitored at Dean Creek marsh over the course of the study to characterize the conditions under which bacterial and fungal assemblages operated. Sapelo Island has a subtropical climate with mild winters and hot summers. Daily temperature maxima in July averaged 32.2°C over a 30-year record (6), and the monthly averages for the two July samples were similar to this long-term average (33.2 and 32.1°C). Daily temperature maxima in January averaged 16.1°C over a 30-year record and averaged 14.3°C during our study. Precipitation was considerably lower than the 30-year average for all sample dates except Jul-01 (Table 1) (6), reflecting a several-year drought in the southeastern United States. Nonetheless, rainfall was highest in July and lower in April and October, as is typical. Microbial communities collected at the two July time points thus experienced considerably hotter and wetter conditions for the preceding months than did communities collected at other time points.

Microbial activity (as measured by respiration rate) peaked in April for *S. alterniflora* blades in the early stage of decay (Table 1), at the same time that live fungal biomass (as measured by ergosterol content) peaked. Microbial communities were equally active on late-decay as on early-decay blades (Mann-Whitney, $P > 0.90$), but respiration rates were less variable for late-decay communities over the 13 months of the study (coefficient of variation was 75% for early-decay respiration rates and 37% for late-decay respiration rates), possibly reflecting a more consistent moisture regime for sediment-associated blades. The average amounts of accumulated fungal biomass were similar for both stages of decay (Mann-Whitney, $P > 0.35$) and varied 2.2-fold during the course of the study (Table 1). Bacterial biomass accumulation was not measured. Overall, microbial communities present at the April time point appeared to be most abundant and active, but considerable microbial activity occurred throughout the year.

The microenvironment of the *S. alterniflora* blades shifted predictably with decomposition stage. Early-decay blades had a higher C/N ratio, higher organic matter content, and lower ash content than late-decay blades (Mann-Whitney; $P < 0.05$, $P < 0.01$, and $P < 0.01$). These data conform to the suggestion that organic density can be used as an indicator of decomposition stage (i.e., higher in younger plant material), while ash content is an indicator of decay-induced infiltration of clay sediment (i.e., higher in older material) (29). An examination of temporal patterns in organic density and ash content indicates that

senesced plant material was most dense and probably least degraded in April and least dense and probably most degraded in July (Table 1).

Fungal community. A database of ITS sequences from 35 fungal isolates and 52 environmental clones obtained from Jul-00 decaying *S. alterniflora* samples was established previously (4) and was used to identify dominant T-RFLP peaks generated in this study (Fig. 1). T-RFLP analysis of fungal communities yielded a total of 20 distinct terminal restriction fragments (T-RFs). Twelve of these fragments could be assigned to a clone or isolate based on empirical evidence (4), and all of the dominant fragments could be assigned.

T-RFs representing the fungal species *Phaeosphaeria spartinicola*, *P. halima*, and *Mycosphaerella* sp. strain 2 were present in all samples analyzed, and environmental isolate "4clt" was present in 70% of the samples. These organisms yielded the most common ITS amplicons, together accounting for 88% ± 9% of the T-RFLP chromatogram area in any given sample (Fig. 2). *Mycosphaerella* sp. strain 2 is comprised of two morphologically cryptic strains (designated group A and group B) and a clone sequence (SIF32) (Fig. 1), all with distinctive T-RFs (144, 410, and 424 bp) (4). Abundance of these peaks was positively correlated (Spearman rank; $r \geq 0.49$; $P \leq 0.008$), and therefore the three fragments were pooled for subsequent analyses.

In most cases, the relative contributions of T-RFs representative of the four dominant fungi were quite similar among the three replicate plots sampled for a given time point and decay stage. In contrast, blades from the two stages of decomposition had significant relative differences in representation of some fungal taxa. *P. halima* relative abundance in the T-RFLP profiles was higher in early compared to late-decay blades (14% versus 9%; *t* test, $P = 0.02$) and "4clt" relative abundance was higher in late compared to early (13% versus 5%; *t* test, $P = 0.04$).

Analysis of ascospore expulsion provided a similar picture of fungal community composition on decaying *S. alterniflora* blades as did the analysis of amplified ITS sequences. The three ascomycetous fungi that yielded the majority of ITS amplicons in the clone library (4) and in the T-RFLP analyses of blades over five seasons also expelled the greatest number of ascospores. *P. spartinicola* tended to be the most prolific expeller, followed by *Mycosphaerella* sp. strain 2, and *P. halima* (Table 1).

Differences in the rate of ascospore expulsion by individual species of fungi associated with blades of the two decomposition stages were common. For example, *P. spartinicola* ascospores were typically a larger percentage of the total ascospores expelled on early- compared to late-decay blades, with a significant difference apparent in the Apr-01 samples (*t* test; $P \leq 0.001$). Alternatively, *P. halima* ascospores were expelled more often from the late-decay blades, with significant differences evident in the Apr-01 and Jul-01 samples (*t* test, $P \leq 0.006$). Expulsion rates for individual species were typically quite variable within the replicate plots, with the coefficient of variation ranging between 17 and 100%. Ergosterol concentration was correlated with the total number of ascospores expelled (Spearman rank, $r = 0.527$, $P = 0.0297$).

Bacterial community. Partial sequences were obtained for 47 cloned 16S rRNA genes from an early-decay library and 37 cloned genes from a late-decay library constructed from the Jul-00 sample. Coverage of amplicon diversity in the 16S rDNA libraries (i.e., percent of amplicon diversity represented) was 38% (early-decay library) and 32% (late-decay library) when the criterion used to define uniqueness was <99% identity (34). Coverage increased to 60% (early-decay library) and 38% (late-decay library) when the criterion for uniqueness was set at <97% sequence identity.

The vast majority of clones sequenced from both the early- (81%) and late-decay (65%) libraries fell within the α-*Proteobacteria* (Fig. 3A). Other taxa represented in both libraries were γ-*Proteobacteria* (4% of early-decay and 14% of late-decay; Fig. 3B), gram-positive group (8.5% of early decay and 8% of late decay; Fig. 3C), and the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group (4% of early decay and 11% of late decay) (Fig. 3D). In addition, one clone sequence (SIB42) from the early-decay library grouped within the planctomycetes, showing 93% identity to a clone from an Australian arid soil sample (GenBank accession no. AF234144). Finally, one clone from the late-decay library (LIB62) was affiliated with the ε-*Proteobacteria*, showing 80% identity to a soil clone (GenBank accession no. AF010040).

For comparative purposes, a collection of culturable marine bacteria associated with the decaying *S. alterniflora* was also assembled. Strains were cultivated from early- (45 isolates) and late-decay (44 isolates) blades as well as from the ascomata of two fungal species known to be important secondary producers in the system, *P. spartinicola* and *Buergenerula spartinae* (9 isolates) (4, 26). As was seen with the clone libraries, the culture collections primarily contained members of the α-*Proteobacteria*, which made up 82% of the early-decay collection, 39% of the late-decay collection, and 89% of the ascomata-associated collection (Fig. 3A). Isolates belonging to the γ-*Proteobacteria* (2% of early-decay and 5% of late-decay collections; Fig. 3B), gram-positive group (13% of early-decay, 20% of late-decay, and 11% of ascomata-associated collections; Fig. 3C), and CFB group (2% of early-decay and 36% of late-decay collections; Fig. 3D) were also cultivated.

Clones and/or isolates were considered to belong to the same operational taxonomic unit (OTU) if they were ≥99% identical over the region of the 16S rRNA gene sequenced (typically 400 bp starting at position ~50). In three instances, we found OTUs that included both clone and isolate sequences. An OTU that was affiliated with *Erythrobacter* was comprised of eight strains from the early-decay isolate collection, one clone from the early-decay library, and three clones from the late-decay library. An OTU that also fell within the *Sphingomonadaceae* contained a clone from the early-decay library and an isolate from the late-decay collection (Fig. 3A). Finally, an OTU within the CFB consisted of an early-decay clone and a late-decay isolate (Fig. 3D).

Bacterial communities yielded 19 major T-RFs. Over half (a total of 11) of these peaks could be tentatively assigned to clones or isolates based on empirically determined *Cfo*I T-RF lengths. Six of the seven most abundant T-RFs were assigned to α-*Proteobacteria* (α-56, α-79, α-231, α-346, α-369, and α-517 bp); the remaining T-RF was indicative of bacteria in the CFB group (C-92) (Fig. 3A). Together, these fragments accounted for 86% ± 13% of the total peak area of any given sample.

One T-RF representing a large group of clones and isolates

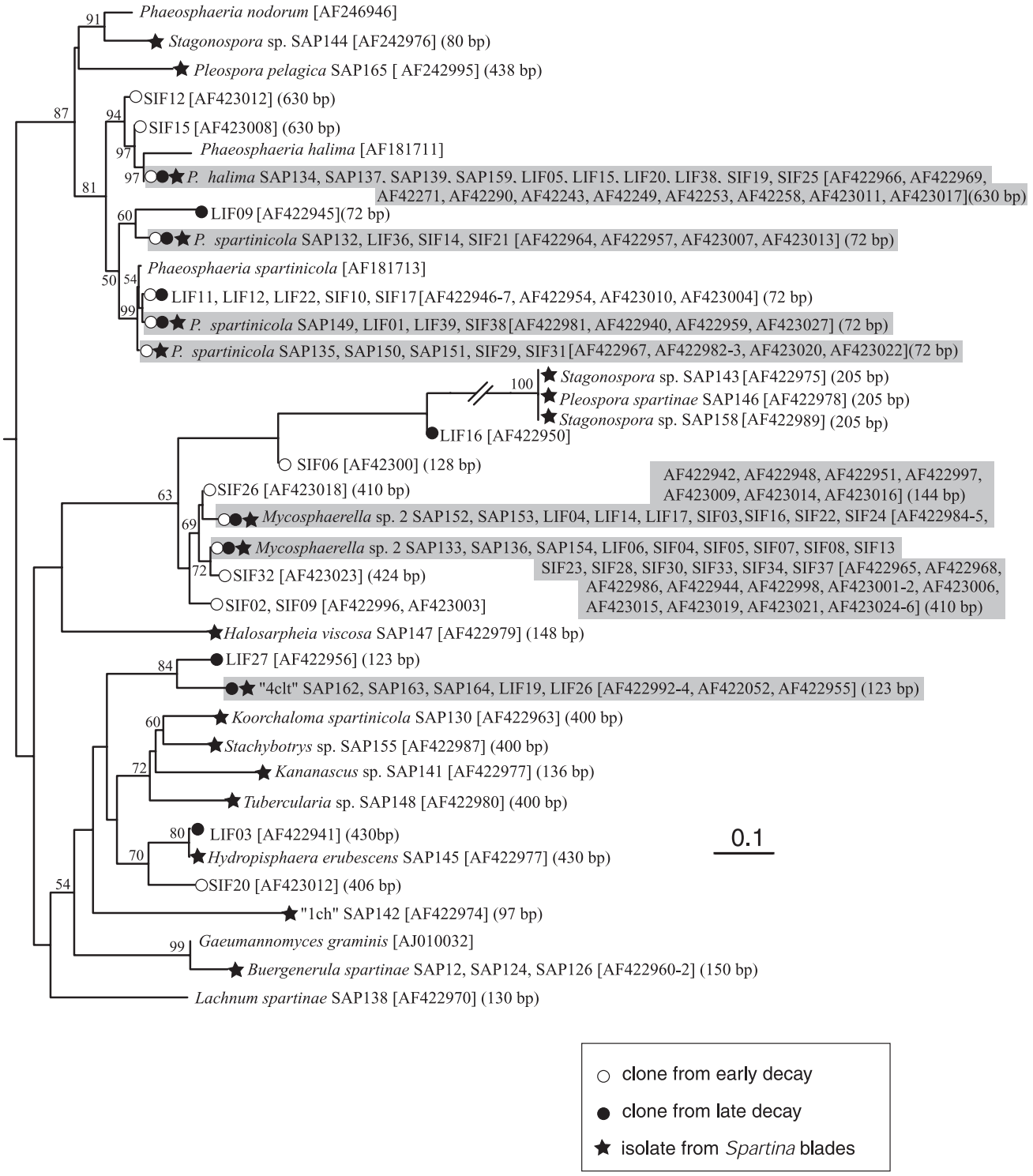

FIG. 1. Phylogenetic tree of ascomycete ITS sequences from cultured strains and PCR amplicons from decaying *S. alterniflora* blades (Jul-00 sample) (4). Sequences are labeled as follows: SAP, ascomycete isolates from Sapelo Island; NRRL, yeast isolates from the National Center for Agricultural Utilization Research; SIF, ITS amplicons from early-decay blades from Sapelo Island; LIF, ITS amplicons from late-decay blades from Sapelo Island. The tree was constructed over 350 positions (ITS1, 5.8S rRNA gene, and ~30 bp of ITS2) using the PHYLIP program and *Scutellospora castanea* (a zygomycete) as the outgroup. Bootstrap values (1,000 resamplings) of >50% are indicated at branch nodes. Empirically determined T-RFs are shown in parentheses. Shaded sequences indicate cases in which a cultured fungus and a clone have >99% sequence similarity. The bar represents Kimura distance. GenBank accession numbers are shown in brackets.

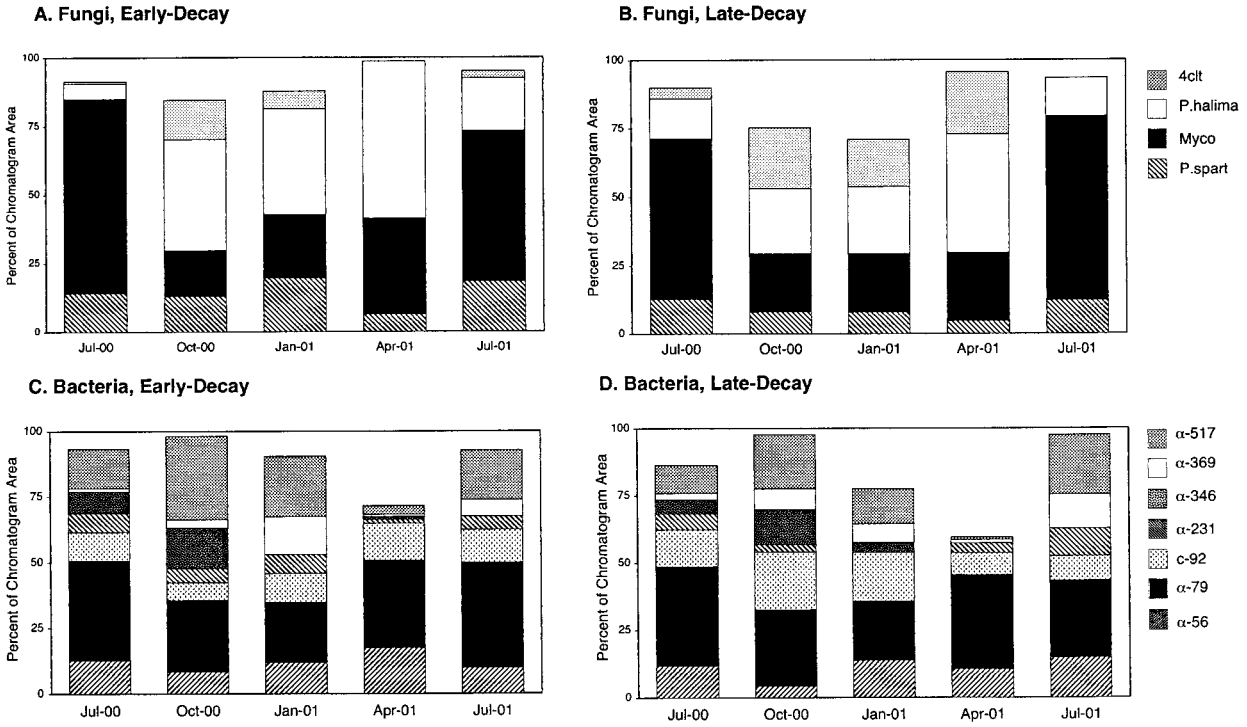

FIG. 2. The relative contributions of the T-RFs of the four major fungal (A and B) and six α-proteobacterial (C and D) groups identified in profiles of microbial communities associated with two different decay stages of *S. alterniflora* blades. The bar heights represent averages from three replicate plots. Myco, *Mycosphaerella* sp. strain 2 (all subgroups); P.spart, *P. spartinicola*.

related to members of the α-proteobacterial genera *Erythrobacter* and *Agrobacterium* (α-79) was found in every sample analyzed and accounted for 31% ± 12% of the chromatogram area (Fig. 2). The T-RF representing the CFB group (C-92) was also present in all samples, where it averaged 13% ± 9% of the total area. T-RF α-56, representing a group of clones and isolates within the *Roseobacter* group, was present in all but one sample (plot 3 late decay, Oct-00) and typically accounted for 11% ± 5% of the total peak area. In some cases, the same T-RF was found in several different α-proteobacterial clades and therefore could not be considered taxonomically informative at the species or genus level (e.g., T-RFs α-231 and α-517; Fig. 3A). Statistical analyses revealed that relative T-RF abundance was not significantly different in blades from the two decomposition stages for any of the seven dominant bacterial taxa (*t* test, $P \geq 0.43$ for all).

Patterns and associations of fungi and bacteria. The bacterial and fungal T-RFs for each sample were pooled and subjected to PCA to discriminate community patterns among samples. Strong seasonality was evident in the analysis (Fig. 4). The three replicate plots for a given season and decay stage generally clustered together, with similar fragments present in the T-RFLP chromatograms and similar relative peak areas for the fragments (i.e., the relative representation of that sequence in the total 16S rDNA or ITS amplicon pool). T-RFs that were present in only one of the three replicate plots typically contributed <5% of the total peak area for that sample. With the exception of the winter (Jan-01) samples, the early- and late-decay samples from a given season also tended to cluster together in the PCA, and PCA loadings indicated that both bacterial and fungal taxa contributed to the seasonal pattern observed (Fig. 4). Differences between the two decay stages in the Jan-01 samples could be attributed to both bacterial and fungal T-RFs (Fig. 5) and included peaks that were not characteristic of other seasons (e.g., fungal Unk-155, bacterial γ-202). Finally, the two July samples (collected 1 year apart) had very similar bacterial and fungal T-RFLP profiles.

To identify the taxa driving the temporal changes in microbial community composition, we examined patterns of abundance of the four fungal taxa and seven bacterial taxa that were most abundant in the T-RFLP profiles. T-RF relative peak area was significantly different among sample dates for all four dominant fungal taxa (analysis of variance [ANOVA], $P \leq 0.04$). Amplicons from *Mycosphaerella* sp. strain 2 (all three T-RFs pooled) had highest relative abundance in the two July samples (65 and 61% of the total peak area) and played a lesser role in the nonsummer samples (averaging 24%). In contrast, *P. halima* and "4clt" amplicons were relatively less abundant in the Jul-00 and Jul-01 samples and more abundant in nonsummer samples (July averages of 13% and 2% for *P. halima* and "4clt," compared to nonsummer averages of 38 and 14%; Fig. 2).

Among the seven bacterial taxa examined, three showed no temporal differences in relative abundance of T-RFs (α-79, C-92, and α-231; ANOVA, $P \geq 0.10$). Of the remaining four, one taxon represented by T-RF α-56 was significantly lower in abundance in the amplicons from the October sample compared to other dates (6% versus 13% of the total peak area); one taxon represented by α-517 was significantly lower in abundance in the April sample than the other dates (2% versus

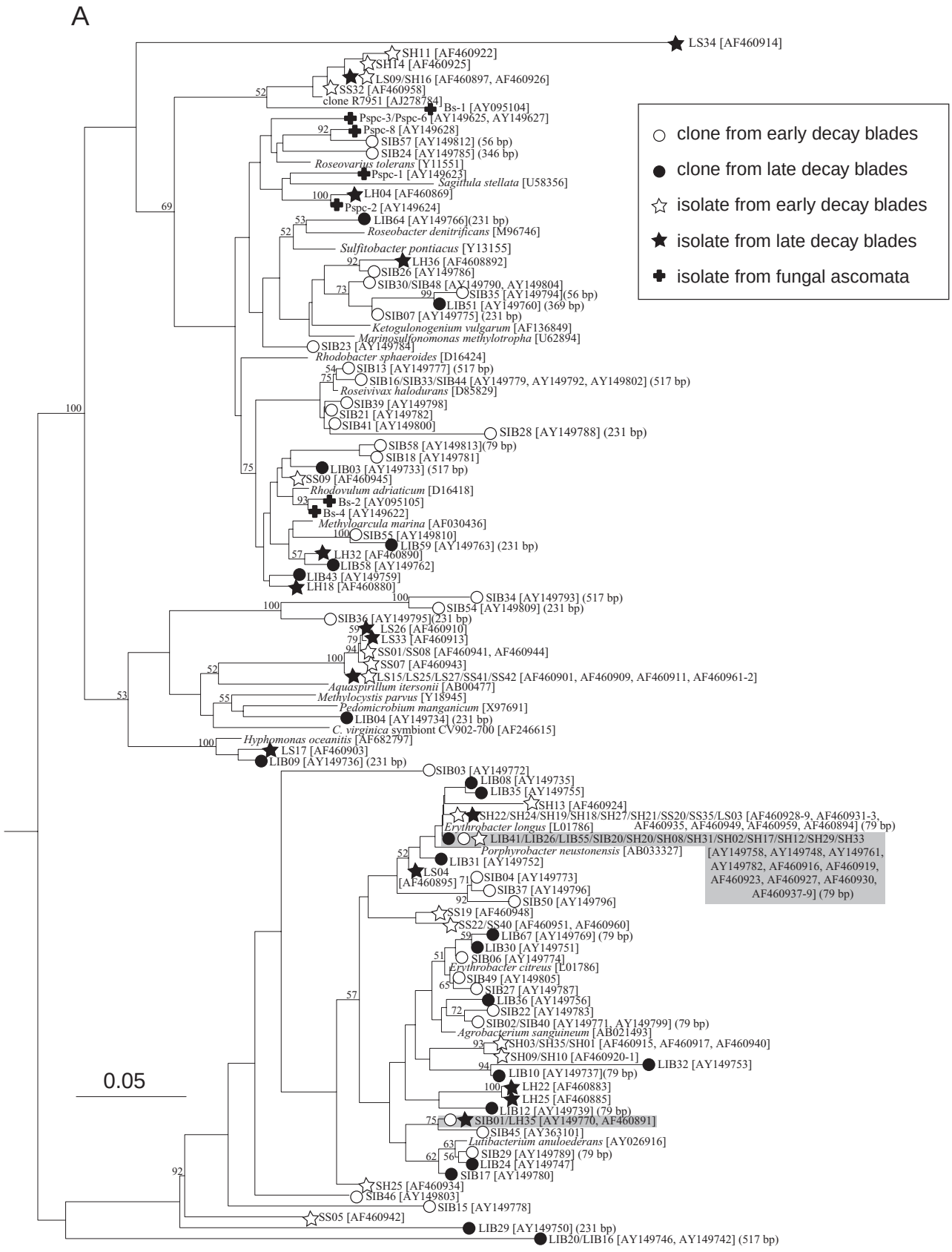
A
clone from early decay blades
clone from late decay blades
isolate from early decay blades
isolate from late decay blades
isolate from fungal ascomata
LS34 [AF460914]
SH11 [AF460922]
SH14 [AF460925]
LS09/SH16 [AF460897, AF460926]
SS32 [AF460958]
clone R7951 [AJ278784]
Bs-1 [AY095104]
Pspc-3/Pspc-6 [AY149625, AY149627]
Pspc-8 [AY149628]
SIB57 [AY149812] (56 bp)
SIB24 [AY149785] (346 bp)
Roseovarius tolerans [Y11551]
Pspc-1 [AY149623]
Sagittula stellata [U58356]
LH04 [AF460869]
Pspc-2 [AY149624]
LIB64 [AY149766](231 bp)
Roseobacter denitrificans [M96746]
Sulfitobacter pontiacus [Y13155]
LH36 [AF4608892]
SIB26 [AY149786]
SIB30/SIB48 [AY149790, AY149804]
SIB35 [AY149794](56 bp)
LIB51 [AY149760] (369 bp)
SIB07 [AY149775] (231 bp)
Ketogulonogenium vulgarum [AF136849]
Marinosulfonomonas methylotropha [U62894]
SIB23 [AY149784]
Rhodobacter sphaeroides [D16424]
SIB13 [AY149777] (517 bp)
SIB16/SIB33/SIB44 [AY149779, AY149792, AY149802] (517 bp)
Roseivivax halodurans [D85829]
SIB39 [AY149798]
SIB21 [AY149782]
SIB41 [AY149800]
SIB28 [AY149788] (231 bp)
SIB58 [AY149813](79 bp)
SIB18 [AY149781]
LIB03 [AY149733] (517 bp)
SS09 [AF460945]
Rhodovulum adriaticum [D16418]
Bs-2 [AY095105]
Bs-4 [AY149622]
Methyloarcula marina [AF030436]
SIB55 [AY149810]
LIB59 [AY149763] (231 bp)
LH32 [AF460890]
LIB58 [AY149762]
LIB43 [AY149759]
LH18 [AF460880]
SIB34 [AY149793] (517 bp)
SIB54 [AY149809] (231 bp)
SIB36 [AY149795](231 bp)
LS26 [AF460910]
LS33 [AF460913]
SS01/SS08 [AF460941, AF460944]
SS07 [AF460943]
LS15/LS25/LS27/SS41/SS42 [AF460901, AF460909, AF460911, AF460961-2]
Aquaspirillum itersonii [AB00477]
Methylocystis parvus [Y18945]
Pedomicrobium manganicum [X97691]
LIB04 [AY149734] (231 bp)
C. virginica symbiont CV902-700 [AF246615]
Hyphomonas oceanitis [AF682797]
LS17 [AF460903]
LIB09 [AY149736] (231 bp)
SIB03 [AY149772]
LIB08 [AY149735]
LIB35 [AY149755]
SH13 [AF460924]
SH22/SH24/SH19/SH18/SH27/SH21/SS20/SS35/LS03 [AF460928-9, AF460931-3, AF460935, AF460949, AF460959, AF460894] (79 bp)
Erythrobacter longus [L01786]
LIB41/LIB26/LIB55/SIB20/SH20/SH08/SH31/SH02/SH17/SH12/SH29/SH33 [AY149758, AY149748, AY149761, AY149782, AF460916, AF460919, AF460923, AF460927, AF460930, AF460937-9] (79 bp)
Porphyrobacter neustonensis [AB033327]
LIB31 [AY149752]
LS04 [AF460895]
SIB04 [AY149773]
SIB37 [AY149796]
SIB50 [AY149796]
SS19 [AF460948]
SS22/SS40 [AF460951, AF460960]
LIB67 [AY149769] (79 bp)
LIB30 [AY149751]
SIB06 [AY149774]
Erythrobacter citreus [L01786]
SIB49 [AY149805]
SIB27 [AY149787]
LIB36 [AY149756]
SIB22 [AY149783]
SIB02/SIB40 [AY149771, AY149799] (79 bp)
Agrobacterium sanguineum [AB021493]
SH03/SH35/SH01 [AF460915, AF460917, AF460940]
SH09/SH10 [AF460920-1]
LIB32 [AY149753]
LIB10 [AY149737](79 bp)
LH22 [AF460883]
LH25 [AF460885]
LIB12 [AY149739] (79 bp)
SIB01/LH35 [AY149770, AF460891]
SIB45 [AY363101]
Lutibacterium anuloederans [AY026916]
SIB29 [AY149789] (79 bp)
LIB24 [AY149747]
SIB17 [AY149780]
SH25 [AF460934]
SIB46 [AY149803]
SIB15 [AY149778]
SS05 [AF460942]
LIB29 [AY149750] (231 bp)
LIB20/LIB16 [AY149746, AY149742] (517 bp)
0.05

FIG. 3.

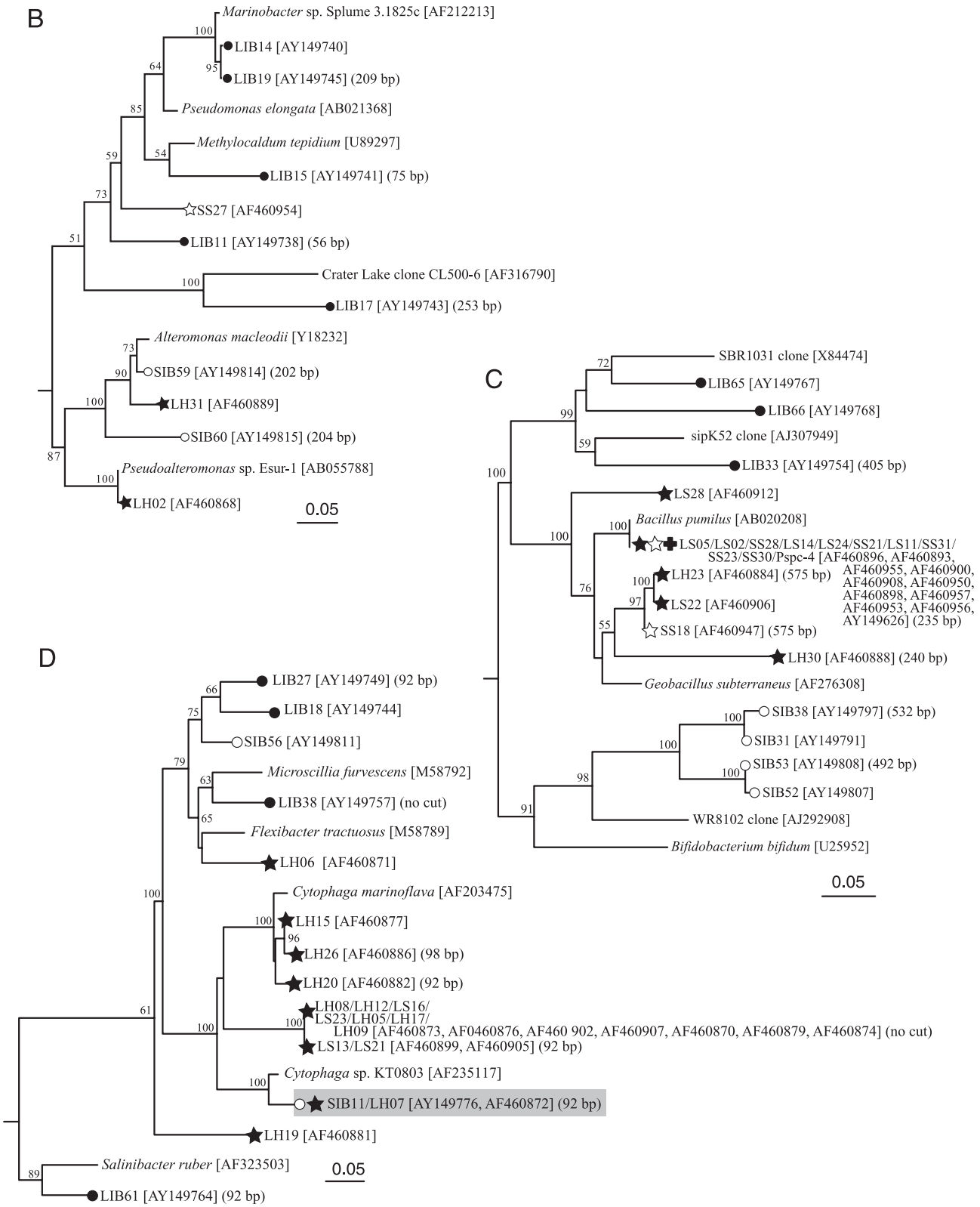

FIG. 3. Phylogenetic tree of 16S rRNA gene sequences from isolates and PCR amplicons from decaying *S. alterniflora* blades (Jul-00 sample) affiliated with α-*Proteobacteria* (A), γ-*Proteobacteria* (B), gram-positive bacteria (C), and *Cytophaga-Flavobacterium-Bacteroides* (CFB) group (D). The tree is based on 300 positions beginning at bp 50 according to the *Escherichia coli* numbering system (J01859) and is unrooted with *E. coli* (A, C, and D) or *Agrobacterium sanguineum* (B) as the outgroup. Bootstrap values (1,000 resamplings) of >50% are indicated at branch nodes. Empirically determined T-RFs are shown in parentheses. Shaded sequences indicate cases in which a cultured bacterium and a clone have >99% sequence similarity. The bar represents Jukes-Cantor distance. GenBank accession numbers are shown in brackets.

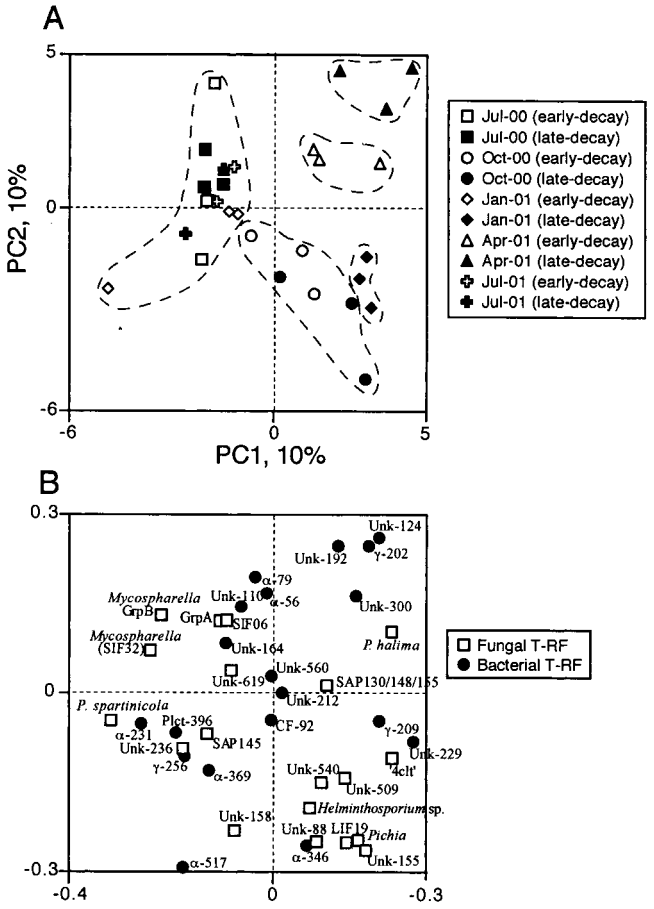

FIG. 4. Principal component plots (PC1 × PC2) of scores of individual samples (A) and loadings of T-RFs (B) generated from T-RFLP profiles of fungal and bacterial communities associated with early- and late-decay *S. alterniflora* blades. Input variables were expressed as percentage of total peak area. Fragments were assigned to specific isolates or clones based on empirically determined T-RFs. Unk, unknown; plct, *Planctomyces*; CF, *Cytophaga-Flavobacterium-Bacteriodes*; α, α-*Proteobacteria*; γ, γ-*Proteobacteria*.

19%); one taxon represented by α-346 was in high abundance in 2000 (averaging 11%) but relatively unimportant in 2001 (averaging 1%); finally, one taxon represented by α-369 was variable in abundance throughout the study (ranging from 1 to 10% of the amplicon pool; Fig. 2).

We also examined patterns of occurrence of the four dominant fungal taxa and the seven dominant bacterial taxa to look for evidence of covariation that might signify ecological interactions. Statistically significant associations (Spearman rank correlation) among the dominant fungi included negative correlations of *Mycosphaerella* sp. strain 2 with *P. halima* and with environmental isolate "4clt." Significant associations among the dominant bacterial taxa included both positive and negative relationships, primarily involving α-proteobacterial groups α-517 and α-56 with several other groups (α-346, C-92, and α-231). Finally, significant associations between bacterial and fungal taxa included a positive association between *P. spartinicola* and two α-proteobacterial taxa (α-369 and α-517), a positive association between the fungus "4clt" and α-346, and a negative association between "4clt" and α-56.

DISCUSSION

Molecular analysis of decomposer communities. ITS/16S rRNA clone libraries and T-RFLP analyses may be susceptible to PCR and/or cloning biases that could affect the interpretation of microbial community dynamics. Such biases appear to be minor for ITS-based analyses of fungal diversity, however, since the composition of this community could be independently verified by both microscopy- and culture-based methods (4). For bacterial communities, the type and extent of biases introduced with the molecular analyses are not known. If significant, they could result in undetected taxa as well as skewed representation of detected taxa in the amplicon pools (7, 35). Further, individual bacterial T-RFs do not necessarily map to a single species. For example, the 16S rRNA *Cfo*I restriction site at 517 bp is characteristic of salt marsh α-*Proteobacteria* from the genera *Roseivivax*, *Rhodovulum*, and *Stappia*, and bacteria that share the α-517 T-RF can have 16S rRNA sequence similarities as low as 76%. Similarly, CFB clones with 16S rRNA sequence similarities of 69% produced the same T-RF at 92 bp. In contrast to the bacterial T-RFs, the fungal T-RFs are informative at the species level, and in some cases even distinguish subspecies (i.e., three T-RFs differentiate subgroups within *Mycosphaerella* sp. strain 2).

General spatial and temporal patterns. The microbial communities of the *S. alterniflora* decay system can be characterized as having low spatial variability, moderate variability due to decomposition stage, and significant temporal variability. PCA of the 39 combined bacterial and fungal T-RFs, which provides an integrated overview of microbial community structure, showed clustering of communities from replicate plots (Fig. 5). Sampling of two marshes approximately 15 and 20 km distant from the Dean Creek marsh in December 2001 confirmed that spatial heterogeneity among decomposer communities of *S. alterniflora* is quite low within the coastal Georgia region (data not shown). For some but not all sample points, the PCA distinguished between microbial communities at different decay stages (early versus late).

In contrast to low spatial variability, PCA indicated considerable temporal variability in community composition. With the exception of the Jan-01 sample, all six microbial communities from a given time point (three replicates each of early- and late-decay samples) grouped together in the analysis and were distinct from other sample dates. Communities from the two July samples collected 1 year apart (Jul-00 and Jul-01) clustered in the T-RFLP analysis.

Relative abundance data for individual T-RFs were used to examine temporal patterns at the individual taxon level. Among the fungi, species that increased in abundance in summer (*Mycosphaerella* sp. strain 2) or winter (*P. halima* and "4clt") emerged from the analyses, as well as those with little seasonality (*P. spartinicola*). In accordance with the T-RFLP data, *Mycospherella* sp. strain 2 was previously found to exhibit a summer peak in rates of ascospore expulsion (approximately threefold higher than in winter) (25). Bacterial taxa exhibited less predictable dynamics that did not always appear to be seasonal. For example, T-RFs representing the α-346 group

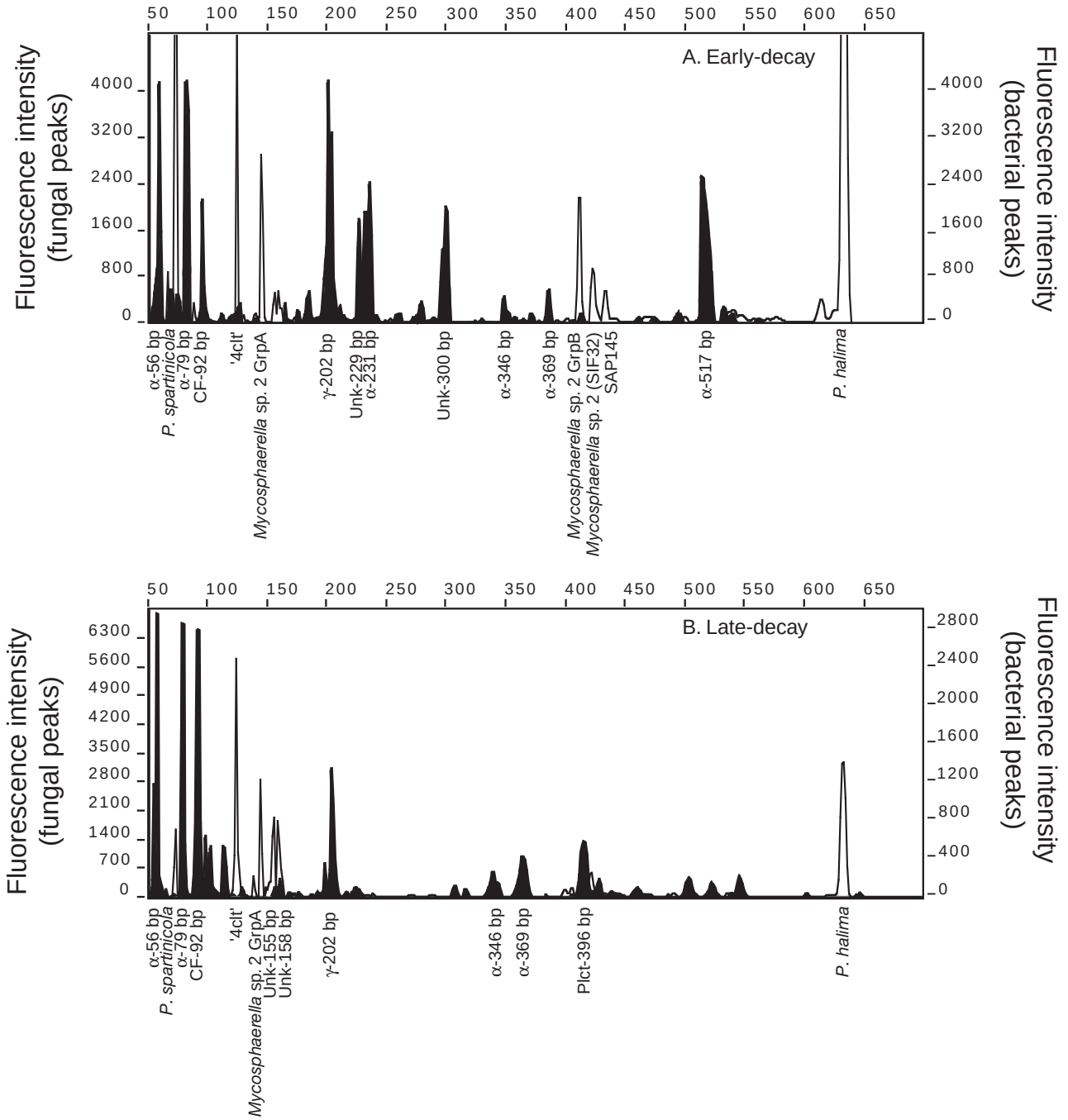

FIG. 5. Overlay of chromatograms of fungal (open peaks) and bacterial (solid peaks) T-RFs for Jan-01 (plot 1) showing early-decay (A) and late-decay (B) blades. Prevalent fragments and/or fragments assigned to specific isolates or clones based on empirically determined T-RFs are indicated beneath the chromatogram. Abbreviations are as described in the legend to Fig. 4.

were in higher relative abundance in samples collected in 2000 than in 2001, while T-RFs of α-56 were significantly less abundant in October relative to July or January. The dynamics of bacterial T-RFs may be complicated by the clustering of multiple species with the same restriction site.

Despite the temporal shifts in relative abundance, there was nonetheless considerable stability over time in the members of the microbial community captured by the T-RFLP analysis. The same four fungal and seven bacterial taxa were represented in almost all samples, with shifts in relative dominance primarily responsible for the observed variability. The fungal community was composed primarily of ascomycetes. Although molecular analyses were limited to ascomycetes (since more general ITS primers retrieved nonfungal sequences) (4), microscopic analyses did not reveal any nonascomycete fungi. Further, previous studies using a variety of methods have

TABLE 2. Contributions of four major bacterial taxa to communities associated with early- and late-decay *S. alterniflora* blades by three methods[a]

Taxon	% of total:					
	Clones in clone library		Isolates in culture collection		Peak chromatogram area by T-RFLP analysis[b]	
	Early	Late	Early	Late	Early	Late
α-*Proteobacteria*	81	65	82	39	78 ± 11	69 ± 10
CFB	4	11	2	36	11 ± 4	14 ± 12
γ-*Proteobacteria*	4	14	2	5	3 ± 1	5 ± 2
Gram-positive bacteria	0	0	13	20	0	0

[a] The methods include cloning and sequencing of amplified 16S rRNA genes, culture on two marine media, and T-RFLP analysis of amplified 16S rRNA genes.
[b] T-RFLP data are the mean of three replicates ±1 standard deviation across five seasonal samples.

shown the fungal community associated with decaying *S. alterniflora* to be strongly dominated by ascomycetes (see reference 26 and references therein). The bacteria in the clone libraries, culture collections, and T-RFLP analyses were strongly dominated by α-*Proteobacteria* (Table 2). Diversity of major groups increased slightly in the late-decay community, however, with a higher representation of *Cytophaga*, γ-*Proteobacteria*, and gram-positive bacteria (isolates only) on the late-decay blades (Table 2).

Analysis of temporal heterogeneity. The pronounced seasonal shifts in microbial community composition (assuming biases from molecular techniques to be constant over time) could be due to responses to the changing composition of the decomposing plant material, responses to various environmental conditions, shifts in microbe-microbe or microbe-predator interactions within the community, or other factors alone or in combination. We examined those taxa with significantly different relative contributions to early- versus late-decay blades and summer versus winter samples for evidence that changing substrate composition might be responsible for microbial community changes, i.e., that both decay stage and seasonal shifts occurred by replacement of species successful on younger detritus (characterized by high C/N, high organic matter density, low ash content) with those successful on more extensively decayed material (low C/N, low organic matter density, high ash content). However, none of the bacterial taxa and only two fungal taxa (*P. spartinicola* and environmental isolate "4clt") showed significant differences in abundance between early- and late-decay blades, and neither of the fungi were more abundant in winter samples. Furthermore, PCA did not indicate clustering of late-decay and late-season samples.

To determine whether seasonally-driven changes in environmental conditions (Table 1) play a role in controlling microbial community composition, T-RFLP abundance for the dominant fungal and bacterial taxa were regressed against temperature, salinity, and rainfall. However, only 1 of 33 tests gave a significant result (the bacterial taxon α-79 was positively correlated with average maximum temperature in the sample month; $r^2 = 0.82$); two significant correlations would be expected by chance alone given the number of comparisons and an α level of 0.05. Although 2000 and 2001 were drought years, the same dominant fungi were found in the *S. alterniflora* decay system in every year of a previous 3-year (1996 to 1999) seasonal study (during which annual rainfall was normal; 80 to 147 cm), suggesting that the microbial community observed here was not anomalous (24, 25). Previous research has suggested that decomposers of *S. alterniflora* shift from a fungus-dominated to a bacterium-dominated community in response to changing substrate composition, juxtaposition to sediment surfaces, and moisture (27, 30). Although it is likely that physical and chemical factors such as detritus age, moisture, temperature, nutrients, and salinity regime indeed have an effect on the composition of the decomposer community, we were unable to link observed shifts in community composition to these parameters in a simple manner.

Associations of microbial taxa. Positive or negative interactions between co-occurring taxa (e.g., in the acquisition of nutrients, in the sequential attack of substrate, in deterring of predators, or in competition for space on the plant blade) may affect microbial community composition (15, 36), as might selective predation of specific taxa by higher trophic levels in the microbial food web (e.g., the selective removal of *P. spartinicola* over *B. spartinae* by invertebrates associated with *S. alterniflora*) (25). We examined the potential importance of microbe-microbe interactions within the *S. alterniflora* decomposer community by exploring statistical associations between taxa. Although such associations may simply reflect similar responses to the same environmental factor (or combination of factors) or may be unrelated to decomposition, they might also be indicative of ecological interactions between bacteria and fungi that would warrant future study. Of the 55 possible correlations between the four dominant fungal and seven dominant bacterial taxa, 11 statistically significant correlations were found; less than 3 such correlations would be expected by chance alone given an α level of 0.05, suggesting that physical associations among microbes in the salt marsh decomposer community occur fairly commonly.

The two significant fungus-fungus interactions both involved negative associations of *Mycosphaerella* sp. strain 2 with other dominant fungi, *P. halima* and environmental isolate "4clt." This may be an indication that *Mycosphaerella* sp. strain 2, which is commonly observed as ascomata closely associated with ascomata of *P. spartinicola*, suppresses competitors in a mutualism with *P. spartinicola* (25, 26). Bacterium-bacterium interactions are more difficult to interpret because each bacterial T-RF can represent a number of related taxa with relatively low 16S rRNA similarities (Fig. 3) (9). Thus, it is not clear which of the several possible taxa represented by one T-RF are statistically related to which of the taxa represented by the other. Nonetheless, all significant bacterial associations involved either α-517 (a large taxon containing clones from several related α-proteobacterial genera) or α-56 (members of the *Roseobacter* clade).

The bacterial-fungal associations involved *P. spartinicola* or environmental isolate "4clt," and occurred with either α-517, α-56, or two other α-proteobacterial groups. It is notable that we found more significant positive (three) than negative (one) bacterial-fungal associations; Gulis and Suberkropp (15) found only antagonistic or competitive interactions between stream bacterial isolates and a common species of freshwater fungus, and Mille-Lindblom and Tranvik (21) report only antagonistic interactions between bacteria and fungi on decomposing

Phragmites litter. Future studies of the complex decomposer communities in southeastern U.S. salt marshes should consider the potential mechanisms of microbe-microbe interactions and the extent to which biotic factors interact with physical and chemical factors to determine the community composition and the fate of vascular plant carbon in this ecosystem. Microbial taxa that may be of particular interest for studying microbe-microbe interactions among the *S. alterniflora* decomposers are the fungi *P. spartinicola* and "4clt" and the bacterial groups represented by T-RFs α-517 and α-56.

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